Exhibit O

PHYTOTHERAPY RESEARCH
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Pycnogenol® reduces Talc-induced Neoplastic Transformation in Human Ovarian Cell Cultures

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Talc and poor diet have been suggested to increase the risk of developing ovarian cancer; which can be reduced by a diet rich in fruit and vegetables. Talc is ubiquitous despite concern about its safety, role as a possible carcinogen and known ability to cause irritation and inflammation. It was recently shown that Pycnogenol® (Pyc; a proprietary mixture of water-soluble bioflavonoids extracted from French maritime pine bark) was selectively toxic to established malignant ovarian germ cells. This study investigated talc-induced carcinogenesis and Pyc-induced chemoprevention. Normal human epithelial and granulosa ovarian cell lines and polymorphonuclear neutrophils (PMN) were treated with talc, or pretreated with Pyc then talc. Cell viability, reactive oxygen species (ROS) generation and neoplastic transformation by soft agar assay were measured. Talc increased proliferation, induced neoplastic transformation and increased ROS generation time-dependently in the ovarian cells and dose-dependently in the PMN. Pretreatment with Pyc inhibited the talc-induced increase in proliferation, decreased the number of transformed colonies and decreased the ROS generation in the ovarian cells. The data suggest that talc may contribute to ovarian neoplastic transformation and Pyc reduced the talc-induced transformation. Taken together, Pyc may prove to be a potent chemopreventative agent against ovarian carcinogenesis. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: ovarian cancer; talc; Pycnogenol®; human neutrophils.

INTRODUCTION

Ovarian cancer is the sixth most commonly occurring cancer and ranks fifth in cancer deaths among women, accounting for more deaths than any other cancer of the female reproductive system. Epidemiological studies have suggested that diet, talc, industrial pollutants, smoking, asbestos and infectious agents may increase the risk of developing ovarian cancer (American Cancer Society, 2000) and may do so by causing localized inflammation (Ness and Cottreau, 1999). Specifically, talc exposure has been cited as a risk factor because of its similarity to asbestos (Cramer *et al.*, 1999).

Talc is a layered magnesium silicate [$Mg_3Si_4O_{10}(OH)_2$]. It is used in cosmetics (as the primary ingredient in talcum powder), pharmaceuticals (as an excipient in tablets) and in many other industrial applications (Bremmell and Addai-Mensah, 2005). Talc is used medically to induce pleurodesis because of its known ability to cause irritation and inflammation (Holthouse and Chleboun, 2001). Animal studies showed a systemic migration of talc particles to various organs despite route of entry (Henderson *et al.*, 1986; Werebe *et al.*, 1999). Exposure of rat ovaries to talc leads to cyst formation (Hamilton *et al.*, 1984). Talc was also shown to cause superoxide anion generation and release from murine macrophages (Van Dyke *et al.*, 2003). Thus controversy continues to surround the topic of talc, its safety (Janssen, 2004) and its role as a possible carcinogen (Cramer *et al.*, 1999; Wong *et al.*, 1999).

Lifestyle factors are important in the etiology of ovarian cancer and current evidence suggests the risk can be reduced by eating a diet rich in fruit and vegetables, among other lifestyle choices (Hanna and Adams, 2006). For the past 20 years, researchers have proposed that nutritional factors play one of the most important roles in the etiology of human cancer. It is estimated that 35% (range 10–70%) of all cancers are diet related and that consumption of certain fruits and vegetables is inversely associated with the incidence of specific forms of cancer. Past research has indicated that a large number of bioactive components, which proved to be protective on different stages of cancer formation, have been identified in nutrients that are of plant origin (Knasmuller and Verhagen, 2002).

Pycnogenol® (Pyc) is a proprietary mixture of water-soluble bioflavonoids extracted from the bark of French maritime pine (*Pinus maritima* Aiton; currently known as *Pinus pinaster* Aiton). The main constituents of Pyc are phenolic compounds, broadly divided into monomers (catechin, epicatechin and taxifolin) and condensed flavonoids (classified as procyanidins and proanthocyanidins). Pyc is known to possess potent antioxidant activity, it not only scavenges the free radicals but it also enhances the endogenous antioxidant systems (Nelson *et al.*, 1998; Wei *et al.*, 1997). Pyc has also been shown to selectively induce apoptosis in breast cancer cells (Huynh and Teel, 2000) and induce differentiation and apoptosis in human promyeloid leukemia cells (Huang *et al.*, 2005). It was previously

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shown that Pyc selectively induced cell death in established malignant ovarian germ cells *in vitro* (Buz'Zard and Lau, 2004). This study now reports that Pyc prevents talc-induced neoplastic transformation of normal ovarian cells, *in vitro*.

MATERIALS AND METHODS

Reagents and chemicals. Pycnogenol® was supplied by Horphag Research (Geneva, Switzerland). Talc, crystal violet, Giemsa stain, RPMI-1640 medium and other miscellaneous chemicals were purchased from Sigma (St Louis, MO). Polymorphoprep™ was purchased from Greiner Bio-One, Inc. (Longwood, FL). Dulbecco's modification of Eagle's Medium (DMEM), Ham's F-12 medium and penicillin–streptomycin (P-S) were purchased from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). High strength analytical grade agarose was purchased from Bio-Rad (Hercules, CA). Ionagar No. 2 was purchased from Oxoid (London, UK). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) was purchased from Molecular Probes (Carlsbad, CA).

Water soluble extraction of Pycnogenol®. Pyc was incubated at 56 °C for 5 h in double distilled water, allowed to cool to room temperature and filtered using a Steriflip® Vacuum Filtration System (0.22 µm Durapore PVDF membrane; Millipore Corporation, Bedford, MA).

Cell culture and treatments. Two cell cultures of human origin were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. OSE2a (immortalized normal ovarian epithelial) and GC1a (immortalized normal ovarian granulosa) cell cultures were donated by Dr Hitoshi Okamura at Kumamoto University, Japan (Okamura *et al.*, 2003). The cell lines were maintained in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 10% FBS and 100 IU/mL P-S. In preparation for either talc or Pyc + talc treatments, each cell line was seeded (1×10^5 cells/ml) and grown to 80% confluence, unless otherwise specified. Cells were incubated with 0–500 µg/mL talc from 24 to 120 h; or 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 24 or 72 h.

Neutrophil isolation and culture. Peripheral blood polymorphonuclear neutrophils (PMN) and monocytes were obtained from heparinized venous blood from healthy volunteers (protocol approved by Loma Linda University Institutional Review Board for Human Studies) and isolated by Polymorphoprep™ density gradient centrifugation followed by the hypotonic lysis of erythrocytes. The purity of PMNs was determined by Giemsa staining as greater than 95%. Purified cells were suspended at 5×10^5 cells/mL in RPMI-1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, supplemented with 10% FBS and 100 IU/mL P-S; and treated with varying concentrations of talc for 24 or 72 h. ROS generation was detected as detailed below.

Cell viability assay. The CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to measure cell viability (Buz'Zard and Lau, 2004). The MTS [3-(4,5-dimethylthiazolyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] solution was used according to manufacturer's instructions. The absorbance was read at 490 nm using a model 3550 Microplate Reader (Bio-Rad). The percent cell viability was calculated as the absorbance of the treated cells divided by the absorbance of the untreated-control cells multiplied by 100.

Neoplastic transformation assay. A characteristic of cancer cells is their ability to grow and to divide when held in suspension without attachment or with minimal attachment to a rigid surface (Leung *et al.*, 2004). Thus, growth in soft agar demonstrates *in vitro* transformation of cells to their neoplastic counterparts (Morales *et al.*, 2003). After 72 h of incubation in the presence of talc; or in the presence of 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 72 h, cells were collected, washed and suspended in 0.35% agarose at 5000 cells/well and layered on top of a base of 0.5% agar. The plates were incubated at 37 °C in a humidified incubator for 14 days. The cells were stained with 0.005% crystal violet and colonies were counted using an inverted microscope (Cory *et al.*, 1987).

Reactive oxygen species (ROS) detection. Carboxy-H_2DCFDA is a non-fluorescent dye that permeates the cells where it is deacetylated by viable cells to 2′,7′-dichlorofluorescin (DCFH), which is then oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by endogenous hydrogen peroxide (H_2O_2) (Wan *et al.*, 1993). The cells were seeded in Optilux™ 96-well plates (BD Falcon, Bedford, MA) and treated with 0 to 500 µg/mL Pyc for 24 h. H_2O_2 (100 µM) was used as a positive control. Carboxy-H_2DCFDA (5 µM) was added and incubated for 1 h. The fluorescence intensity (excitation 485 nm/emission 530 nm) was measured as arbitrary fluorescent units (AFU) using a model 7600 Microplate Fluorometer (Cambridge Technology, Inc., Watertown, MA). The percent AFU (a.k.a. % ROS generation) was calculated as the 'treated cell-AFU' divided by the 'untreated cell-AFU' multiplied by 100. Immediately following the fluorescence detection, the fluorescence intensity was normalized by the cell viability assay.

Statistical analysis. Data were reported as mean ± SE. Statistical analysis was performed with the Student's paired *t*-test.

RESULTS

All experiments were performed a minimum of three times with reproducible results.

Effect of talc on cell viability of normal ovarian cells

Talc caused a bell-shaped curve response in OSE2a cells, with a statistically significant increase seen at 5 µg/mL (24 h) and a statistically significant decrease at 200 µg/mL (72 h) and 500 µg/mL (24 and 72 h) (Fig. 1).

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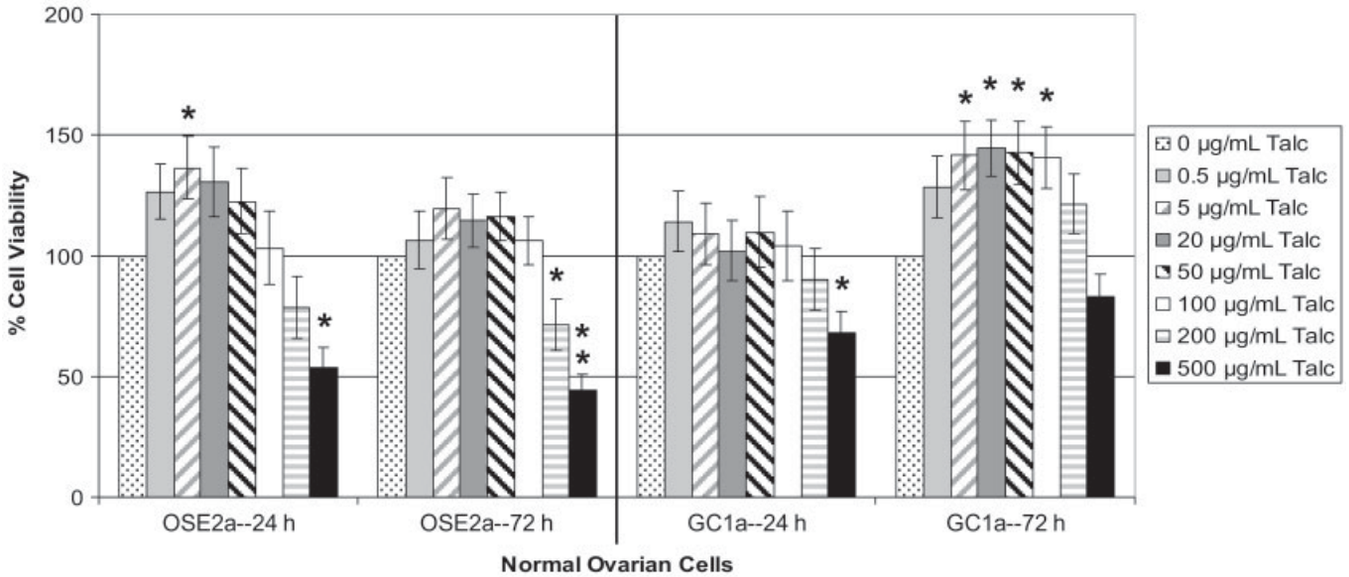

Figure 1. Effect of talc on the cell viability of ovarian cells. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were treated with various concentrations of talc for 24 and 72 h. Cell viability was measured by the MTS assay and the percent cell viability was calculated as the absorbance of the treated cell divided by the absorbance of the untreated-control cells multiplied by 100. Each data point represents mean ± SE of five determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ comparing the treatment with the respective untreated control.

Also seen in Fig. 1, talc caused a bell-shaped curve response in GC1a cells, with a statistically significant increase seen at 5, 20, 50 and 100 µg/mL (72 h) and a statistically significant decrease at 500 µg/mL (24 h).

Effect of talc on neoplastic transformation of normal ovarian cells

Since the ability to grow suspended in soft agar is a characteristic of cells being transformed to their neoplastic counterparts (Leung *et al.*, 2004; Morales *et al.*, 2003), the study determined whether talc would be able to induce such a transformation. As shown in Fig. 2, talc caused a statistically significant increase in the number of transformed colonies in the OSE2a cells at 5 and 20 µg/mL talc and in the GC1a cells at 5, 20 and 100 µg/mL talc, compared with the untreated control. An exception was seen in the 100 µg/mL talc treatment in the OSE2a cells in which the number of transformed colonies was reduced significantly.

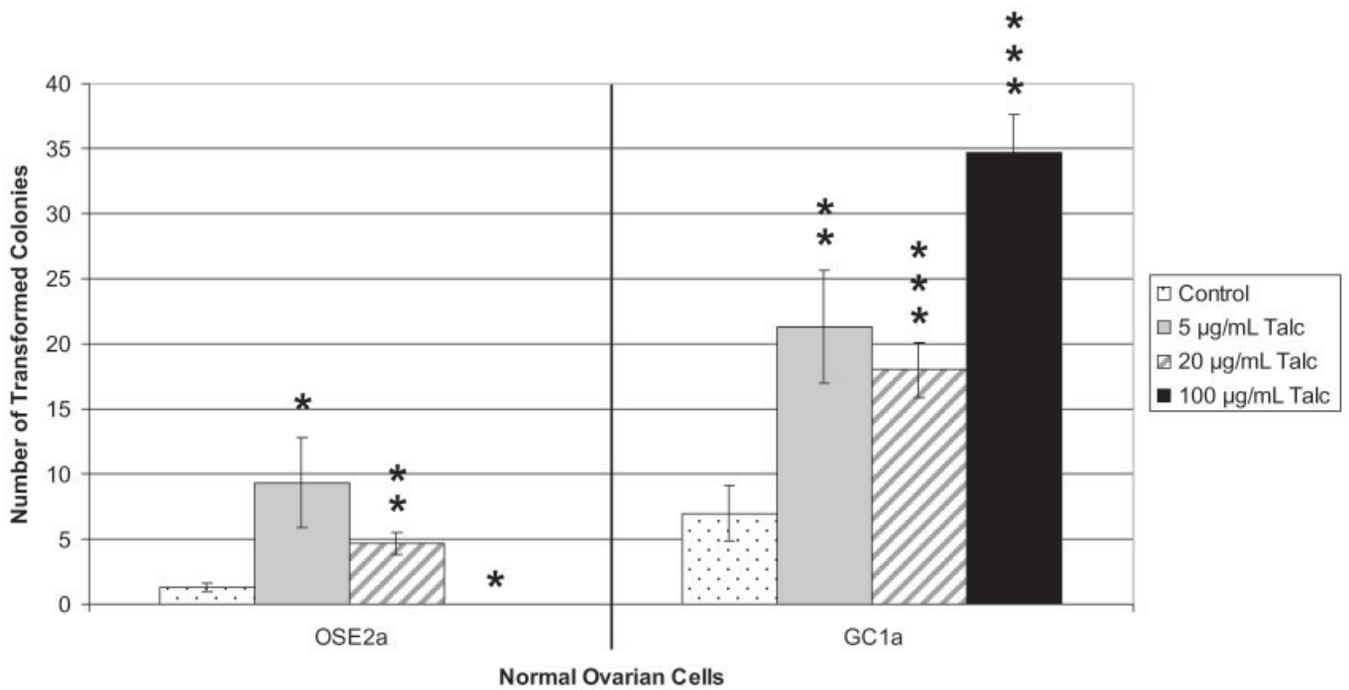

Figure 2. Neoplastic transformation of ovarian cells by talc. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were incubated with various concentrations of talc for 72 h, collected, washed, seeded in soft agar suspension and grown for 14 days before colonies were counted. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control (0 µg/mL talc).

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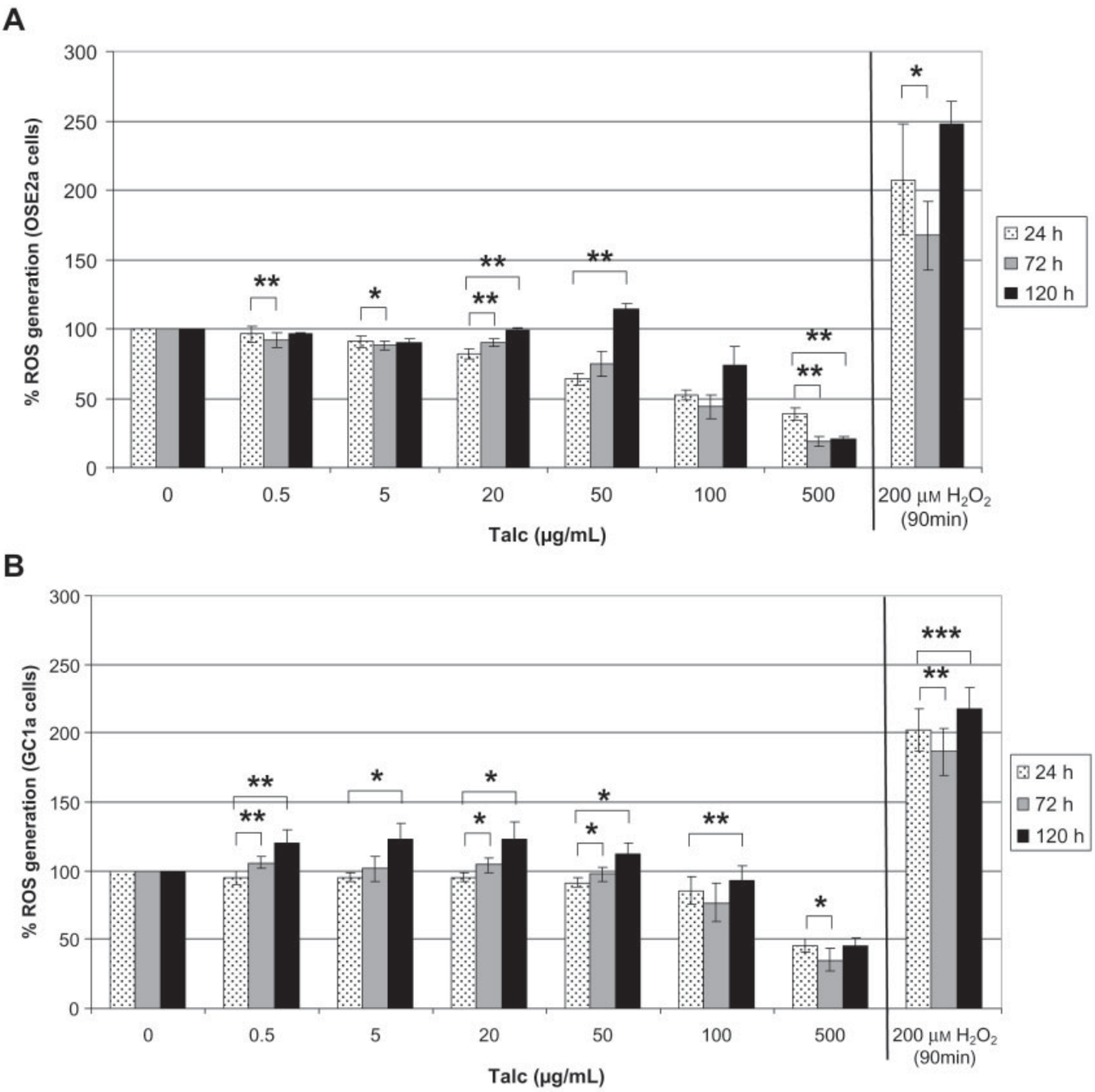

Figure 3. ROS generation of ovarian cells in response to talc treatments. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were treated with various concentrations of talc for 24, 72 and 120 h and H_2O_2 during the last 90 min of each respective time point. H_2O_2 was used as a positive control for this assay. Fluorescence intensity were measured as arbitrary fluorescent units (AFU) at ex 485 nm/em 530 nm and normalized with the cell viability assay. Percent AFU (a.k.a. % ROS generation) was calculated as the average AFU of the treated cell divided by the average AFU of the untreated-control cells multiplied by 100. (A) ROS generation in OSE2a cells in response to talc treatments. (B) ROS generation in GC1a cells in response to talc treatments. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control (as demonstrated by the horizontal brackets).

Effect of talc on ROS generation in normal ovarian cells

Talc caused an initial dose-dependent decrease in ROS generation (24 h) which increased with time in OSE2a cells (Fig. 3A). However, as time increased, ROS generation rebounded and increased compared with the values at 24 h. A statistically significant increase was seen at 20 µg/mL (72 and 120 h) and 50 µg/mL (120 h). Talc also caused an initial dose-dependent decrease in ROS generation (24 h) in GC1a cells (Fig. 3B), but ROS generation increased with time in the talc treated cells. A statistically significant increase was seen with 0.5, 20 and 50 µg/mL (72 and 120 h), as well as 5 and 100 µg/mL (120 h) compared with the respective 24 h value.

Effect of talc on ROS generation in PMN

Since oxidative stress is often a component of the tumor microenvironment (Valko *et al.*, 2004), the study tested whether talc was capable of inducing ROS generation

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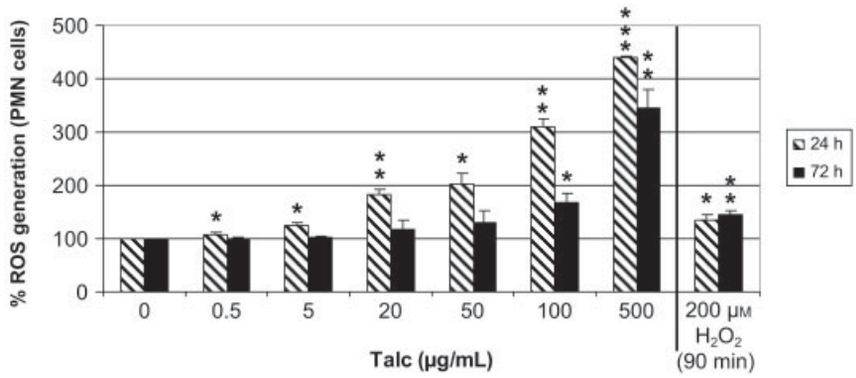

Figure 4. ROS generation of polymorphonuclear neutrophils (PMN) in response to talc treatments. PMNs were treated with various concentrations of talc for 24 and 72 h and H_2O_2 during the last 90 min of each respective time point. H_2O_2 was used as a positive control for this assay. Fluorescence intensity were measured as arbitrary fluorescent units (AFU) at ex 485 nm/em 530 nm and normalized with the cell viability assay. Percent AFU (a.k.a. % ROS generation) was calculated as the average AFU of the treated cell divided by the average AFU of the untreated-control cells multiplied by 100. ROS generation of PMNs in response to talc treatments. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control.

in human PMNs. Talc caused a dose-dependent increase in ROS generation at both time points (Fig. 4). The increase was statistically significant at 0.5, 5, 20, 50 µg/mL (24 h) and 100 and 500 µg/mL (24 and 72 h). The maximum ROS generation was seen at 500 µg/mL and was increased over 4-fold at 24 h and 3.5-fold at 72 h, compared with the respective untreated cells.

Effect of pretreatment with Pyc on talc-induced cell viability changes in normal ovarian cells

Pretreatment with Pyc did not cause a statistically different change in cell viability in the OSE2a cells (Fig. 5A). Pretreatment with Pyc caused a general decrease in cell viability in the GC1a cells (Fig. 5B) compared with the respective untreated GC1a cells. One exception is that of a slight, but statistically significant, increase in cell viability at 100 µg/mL Pyc + 5 µg/mL talc (72 h) compared with the respective untreated GC1a cells (Fig. 5B).

Effect of pretreatment with Pyc on talc-induced neoplastic transformation of normal ovarian cells

Pretreatment with Pyc decreased the number of neoplastically transformed colonies induced by talc in

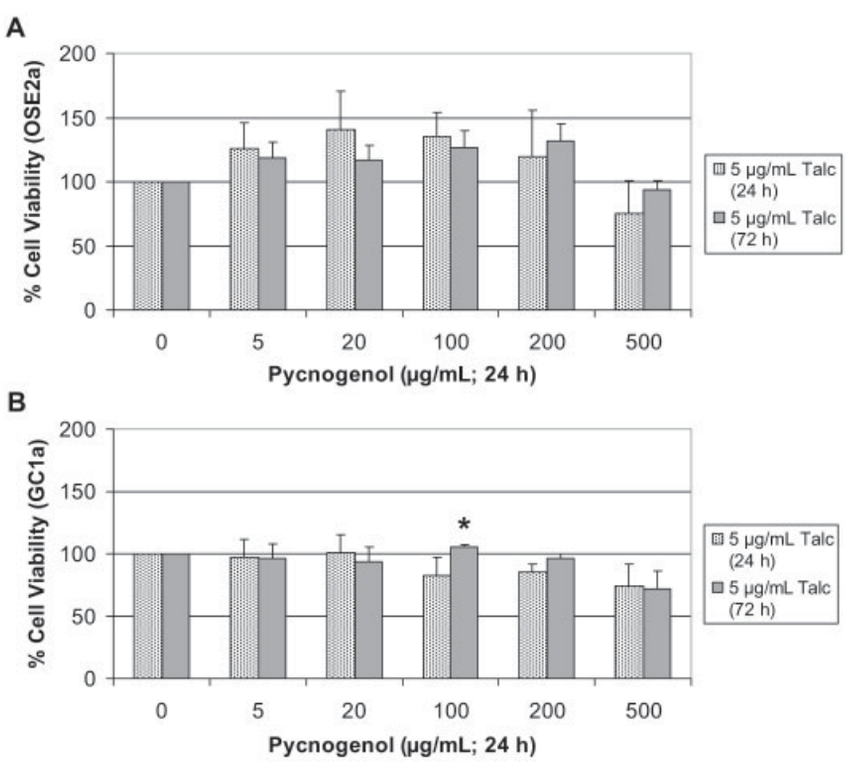

Figure 5. Effect of Pyc + talc treatments on the cell viability of ovarian cells. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were treated with 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 24 and 72 h. Cell viability was measured by the MTS assay and percent cell viability was calculated as the absorbance of the treated cell divided by the absorbance of the untreated-control cells multiplied by 100. (A) OSE2a cells. (B) GC1a cells. Each data represent mean ± SE of four determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$ comparing the treatment with the respective untreated control.

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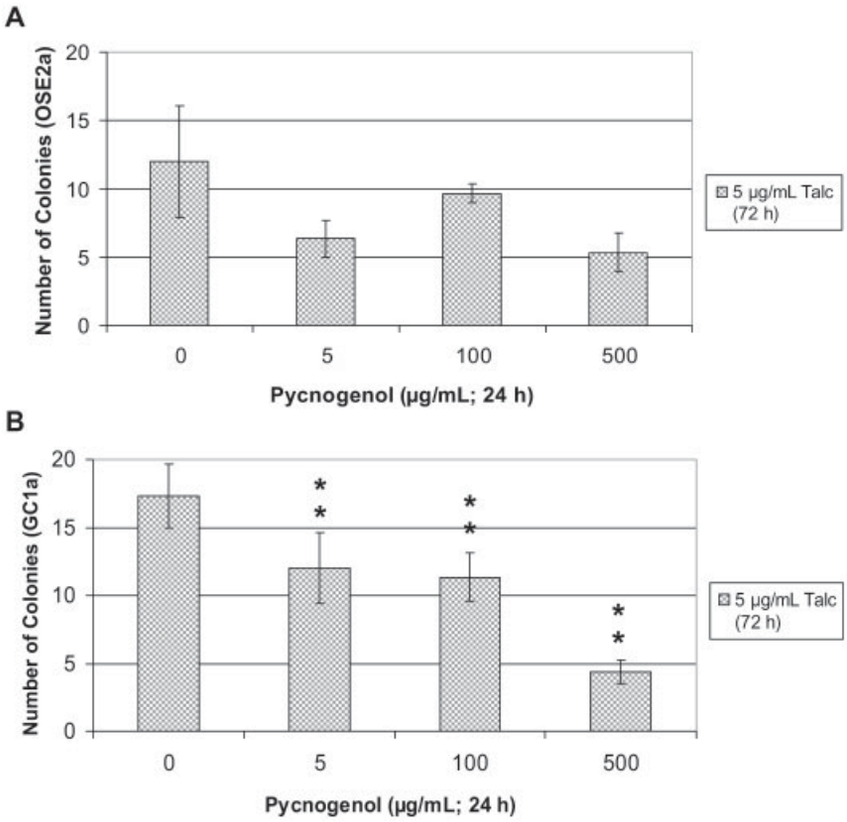

Figure 6. Pyc-induced protection against neoplastic transformation of ovarian cells by talc. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were incubated with 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 72 h, collected, washed, seeded in soft agar suspension and grown for 14 days before colonies were counted. (A) OSE2a cells. (B) GC1a cells. Each data represent mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. ** $p < 0.01$ comparing the treatment with the respective control.

the OSE2a cells, but not in a statistically significant manner (Fig. 6A). Pretreatment with Pyc (5, 100 and 500 µg/mL; 24 h) caused a statistically significant decrease in the number of talc-induced neoplastically transformed colonies in the GC1a cells (Fig. 6B).

Effect of pretreatment with Pyc on talc-induced ROS generation in normal ovarian cells

Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 100 and 200 µg/mL Pyc + 5 µg/mL talc (24 h); and 500 µg/mL Pyc + 5 µg/mL talc (24 and 72 h) in the OSE2a cells (Fig. 7A). Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 200 and 500 µg/mL Pyc + 5 µg/mL talc (24 h) in the GC1a cells (Fig. 7B). Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 100, 200 and 500 µg/mL Pyc + 5 µg/mL talc (72 h) in the GC1a cells (Fig. 7B). The decrease seen at 100 µg/mL Pyc + 5 µg/mL talc (24 h) was not statistically significant (Fig. 7B).

DISCUSSION

Cancer development is a multi-step process comprising a series of cellular and molecular changes that are mediated by various endogenous and exogenous stimuli, such as aberrantly expressed ROS (Storz, 2005). Although ROS are a byproduct of endogenous biochemical processes, ROS (such as H_2O_2) at high concentrations or expressed in a chronic nature can damage cellular macromolecules and contribute to neoplastic transformation and tumor growth (Nicco *et al.*, 2005). A characteristic of neoplastically transformed cells is their ability to grow and to divide when held in suspension without attachment or with minimal attachment to a rigid surface (Leung *et al.*, 2004; Morales *et al.*, 2003). Our data show that talc not only increased cell viability (Fig. 1A), but also caused an increase in transformed cells in both the stromal and epithelial ovarian cells by their ability to grow, divide and form colonies while being suspended in soft agar (Fig. 2A).

It is known that substances that raise the intracellular level of H_2O_2 are able to trigger normal cell proliferation and abolish tumor cell proliferation (Ness and Cottreau, 1999; Nicco *et al.*, 2005). In normal cells, the basal level of H_2O_2 is low and its increase is initially associated with cell growth. H_2O_2 at high concentrations or expressed in a chronic nature in normal cells, can damage cellular macromolecules and contribute to neoplastic transformation and tumor growth (Nicco *et al.*, 2005). In this study, talc was shown to increase the ROS generation, after an initial suppression, in a time-dependent manner in the normal stromal cells (Fig. 3B) and less strongly in the normal epithelial cells (Fig. 3A).

Recent studies have expanded the concept that inflammation is a critical component of tumor progression. The neoplastic process (proliferation, survival and

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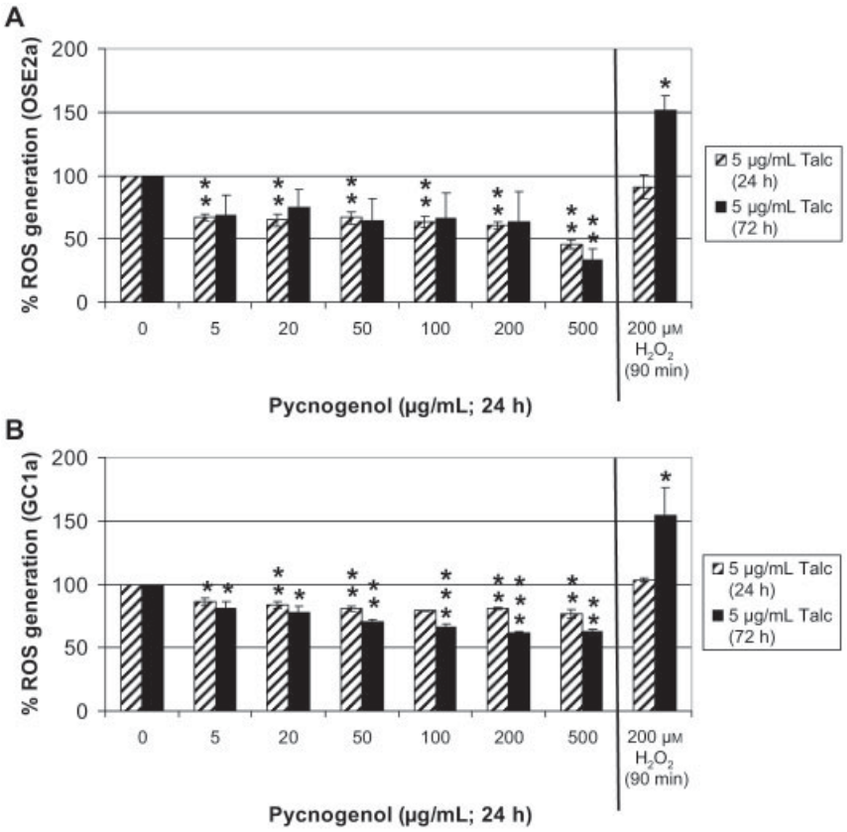

Figure 7. ROS generation of ovarian cells in response to Pyc + talc. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were treated with 0–500 µg/mL Pyc for 24 h, followed by 5 µg/mL talc for 24 or 72 h and H_2O_2 (the last 90 min of each time point) as a positive control. Fluorescence intensity (AFU) was measured at ex 485 nm/em 530 nm and normalized by cell viability assay. The percent ROS generation was calculated as the average AFU of treated divided by AFU of untreated-control multiplied by 100. (A) OSE2a cells. (B) GC1a cells. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control.

migration) is linked with the tumor microenvironment and synchronized with inflammatory cells (Valko *et al.*, 2004). Polymorphonuclear neutrophils and macrophages are a main source of exogenous ROS in that they release large quantities of ROS in response to a variety of stimuli. This exogenously produced ROS is crucial in the innate immune system of the host for killing invading bacteria but may also be responsible for tissue injury, when expressed excessively or inappropriately (Lewis and Pollard, 2006). Inflammatory cells are prominent in the stromal compartment of virtually all types of malignancy. These highly versatile cells respond to the presence of stimuli in different parts of tumors (Balkwill and Mantovani, 2001). In an *in vitro* study of rat cells, both macrophages and neutrophils were found to be mutagenic in response to alpha-quartz dust, talc and diesel soot; however, neutrophils appeared to have a greater mutagenic effect (Driscoll *et al.*, 1997). This study found that talc not only increased the ROS generation in the ovarian cells (Fig. 3), but also increased the expression of ROS by the neutrophils (Fig. 4).

Talc has been shown to be ubiquitous in our modern environment (Bremmell and Addai-Mensah, 2005) despite concerns raised about its safety (Janssen, 2004), its role as a possible carcinogen (Cramer *et al.*, 1999; Wong *et al.*, 1999), and its known ability to cause irritation and inflammation (Holthouse and Chleboun, 2001). The data show that talc is capable of increasing cell proliferation, inducing neoplastic transformation of both the normal stromal and epithelial ovarian cells *in vitro*; and increasing ROS generation in these cells as well as the PMN cells.

Cancer chemoprevention is regarded as an efficient strategy to prevent cancer. The most useful cancer chemopreventive compounds will have minimal long-term toxicity, while significantly reducing tumor incidence, delaying tumor onset or preventing tumor progression (Kapadia *et al.*, 2003). It was hypothesized that Pyc, shown to induce apoptosis in various malignant cells (Huang *et al.*, 2005; Huynh and Teel, 2000), could prevent talc-induced neoplastic transformation of normal ovarian cells. It was recently shown that Pyc selectively induced cell death in established malignant ovarian germ cells *in vitro* (Buz'Zard and Lau, 2004). The present study showed that Pyc was capable of inhibiting the above mentioned talc-induced changes. Pretreatment with Pyc prevented the characteristic talc-induced increase in cell viability of GC1a cells (Fig. 5B). Pretreatment with Pyc was able to decrease the ROS generation compared with the respective controls both in a dose- and time-dependent manner (Fig. 7). The data show that pretreatment with Pyc reduced the number of talc-induced transformed colonies in both cell lines (Fig. 6). In the GC1a cells, the decrease in the number of transformed colonies was statistically significant at all concentrations of Pyc (Fig. 6B).

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In conclusion, our *in vitro* data suggest that: (1) talc may contribute to ovarian carcinogenesis in humans by way of inducing aberrant ROS generation and (2) Pyc reduces talc-induced neoplastic transformation of ovarian cells. Taken together, Pyc may prove to be a chemopreventative agent against ovarian carcinogenesis.

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